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(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF SHEFFIELD** [GB/GB]; Western Bank, Sheffield S10 2TN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **HOLLANDER, Anthony, Peter** [GB/GB]; University of Bristol, Academic Rheumatology Unit, Avon Orthopaedic Centre, Southmead Hospital, Bristol BS10 5NB (GB). **BARKER, Michael, David** [GB/GB]; University of Sheffield, Division of Oncology & Cellular Pathology, Pathology Section, Medical School, Sheffield S10 2RX (GB). **KAFIENAH, Waeh** [PK/PK]; University of Sheffield, Division of Biochemical & Muscoloskeletal Medicine, Section of Human Metabolism & Clinical Biochemistr, y, Medical School, Beech Hill Road, Sheffield S10 2RX (GB).

(74) Agent: **MARKGRAAF PATENTS LIMITED**; The Crescent, 54 Blossom Street, York YO24 1AP (GB).

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(54) Title: CELL TRANSFECTION

(57) Abstract: An isolated nucleic acid molecule comprising at least the functional part of the blasticidin resistance (BS) gene, or homologue thereof, linked via a recognition sequence, to at least one selected gene; a method for the preparation thereof, a method for transfecting cells, a transfected cell and tissue comprising a transfected cell, a method for "knockout", a method for incubating mitotically inactive cells, a therapeutic composition comprising transfected cells or tissue, and the use thereof.

CELL TRANSFECTION

The invention herein described relates to a transfection/transformation method for use in the introduction of nucleic acid molecules into cells; vectors for use in said transfection/transformation method; cells transfected/transformed with said vectors via said method; and cells/tissues transfected/transformed with said vectors via said method for use in, typically, but not exclusively, therapeutic and cosmetic tissue engineering.

Cell transfection/transformation, in the context of genetic engineering, is the introduction of DNA into a cell which alters the genotypic character such that ultimately said transfected/transformed cell has an altered phenotype. A classical example of such a transformation event is shown by the transformation of bacterial cells with DNA containing an antibiotic resistance gene (e.g. β lactamase (ampicillin resistance), chloramphenicol acetyltransferase (chloramphenicol resistance)).

The term transfection is sometimes used interchangeably with the term transformation. In general transformation is the introduction of DNA into a bacterial or fungal cell. Transfection relates to the process by which DNA is introduced into a higher eukaryotic cell. For the sake of clarity the term transfection is used to cover both procedures.

The alteration in phenotype, as a consequence of transfection, may manifest itself in a variety of ways. Typically, by example, and not by way of limitation, the transfection event may introduce a gene which encodes an enzyme not normally present in said cell thus resulting in the acquisition of an enzyme activity not shown by said cell

Bacterial cells were the first cells to be routinely genetically transformed. This was closely followed by lower eukaryotic cells (e.g. *Saccharomyces*

cerevisiae, *Neurospora crassa, Aspergillus nidulans*) and later by higher eukaryotic cells (e.g. insect cells, mammalian cells, plant cells). The introduction of nucleic acid into selected cells has greatly facilitated our understanding of gene function at all levels of control of gene expression. In addition it has also facilitated our understanding of differential gene expression through identification of cell/tissue specific regulators (eg transcription factors, receptors , ligands).

For simplicity, the term transfection will be used hereinafter when referring to the introduction of DNA into cells.

Transfection of cells may be accomplished by either stable transfection (i.e. the transfecting DNA integrates into the genome of the host cell and is replicated and segregated with the host chromosomes during mitosis) or, alternatively, the transfecting DNA, when provided with an origin of replication that will allow its replication in a host cell, can be maintained extrachromasomally via independent replication and segregation to daughter cells during cell division.

A common element in all transfection methodologies is the provision of a selectable marker gene which, under appropriate growth conditions, allows the identification or selection of those cells harbouring the transfected DNA from those cells in which the DNA has not been introduced. For example, and not by way of limitation, in higher eukaryotic cells, a classical resistance gene specific to an aminoglycosidic antibiotic, G-418 sulphate, is aminoglycoside phosphotransferase 3. Typically, during the selection process, stably transfected cells containing DNA encoding aminoglycoside phosphotransferase 3 are exposed to the antibiotic G-418 sulphate in the growth culture. Those cells that have recombined with the transfecting DNA will express the enzyme and remove the drug from the cell thus preventing its

harmful effects on cell function. Those cells not harbouring the transfecting DNA will be sensitive and therefore suffer the consequences of exposure to G-418 sulphate.

Conventionally, nucleic acid molecules used to transfect cells are referred to as vectors. Vectors used in genetic engineering are typically circular molecules, (although some may be linearised prior to transfection to facilitate the introduction of DNA into a host cell). Vectors of this type are referred to as plasmids (eg pBR 322, pUC series,), phages (M13 series, λ phage series) or phagemids (pGEM^{R} series, Promega Corp^{TM}) the latter contains, in addition to a resistance gene, certain other features which facilitate its handling and manipulation. For example, and not by way of limitation, vectors used in genetic engineering typically contain: an origin of replication which allows the vector to replicate and segregate in bacterial cells to provide large quantities of vector DNA for use in genetic engineering experiments; an antibiotic resistance gene which allow selection in said bacterial cell; a, so-called, multiple cloning site which facilitates the introduction of DNA into the vector; and, in some examples, transcription promoter sequences which facilitate the expression of the DNA introduced at the multiple cloning site; and transcription termination sequences and processing sites which ensure RNA transcripts are correctly processed (eg polyadenylation sites). Vectors of the type described above are well known in the art and are extensively described in Sambrook *et al* (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY; Kaiser K and Murray N (1985) DNA Cloning Techniques:A Practical Approach, IRL Press, Oxford; Ausubel, FM *et al* Current Protocols in Molecular Biology John Wiley and Sons, NY.

In addition to the above identified vectors, viral based vectors are used in transfection and in particular, gene therapy, to deliver therapeutic genes to

tissues *in vivo*. These vectors typically retain the capability to infect a host cell but are genetically modified to render the virus biologically disabled, this latter feature facilitates its removal from the organism and prevents its uncontrolled spread through host tissues. Examples of viral based vectors used in gene therapy include by example and not by way of limitation; adenovirus; retrovirus; parvovirus; herpesvirus; adeno-associated viruses and lentiviruses.

There are a variety of methods by which the vectors described above (other than viral based vectors) can be introduced into selected cells. For example, and not by way of limitation, bacterial cells may be made competent for the introduction of a vector after incubation in $CaCl_2$ or RbCl (Chung, C.T., Niemela, S.L., and Miller, R.H. 1989. One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. U.S.A.* 86:2172-2175). Bacterial cells treated in this way become permeable to high molecular weight vectors which facilitates their introduction into the cell.

Fungal cells have an outer cell wall which is impermeable to high molecular weight molecules. However, incubation of yeast cells in LiCl or LiOAc does render the yeast cell wall permeable to vectors (Ito, H., Fukuda, Y., Murata, K., and Kimura, A. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153:163-168). In addition, yeast cells may be treated with a variety of cell wall digesting enzymes which remove the outer cell wall, thereby making the yeast cell permeable to large molecular weight vectors (Beggs, J.D. 1978. Transformation of yeast by a replicating hybrid plasmid. *Nature (Lond.)* 275:104-109).

Higher eukaryotic cells may also be transfected via a variety of techniques. For example, and not by way of limitation, DNA may be introduced into mammalian cells via calcium phosphate precipitation (Graham, FL and Van

der Eb AJ, (1973) Virology 52, p456). This technique is particularly useful for both transient and stable transfection. An alternative to calcium phosphate precipitation is DEAE dextran mediated transfection (Gluzman, Y. (1981) Cell, 23, 175). This method is used primarily for transient transfection rather than stable transfection.

More recently, both prokaryotic and eukaryotic cells have been transfected using a pulse of high voltage electricity which when passed through a culture of cells, in the presence of vector DNA, momentarily results in permeabilisation of the cell membrane thus facilitating the introduction of vectors into said cells. This procedure is referred to as electroporation.

Transfection can also occur through direct injection of nucleic acid into a cell. An example of this is provided by the production of transgenic organisms which will be further described below.

Clearly, in the last 30 years, the development of vectors for use in the transfection of cells has rapidly evolved in accordance with methods via which said vectors may be introduced into cells.

Our studies have addressed a problem regarding the transfection of cells, typified by, but not exclusive to, differentiated cells/tissues. Differentiated cells/tissues are particularly difficult to transfect and maintain in culture in a differentiated state. In addition those cells that are transfected with a selected gene do not show a consistent pattern of expression.

Our experiments have used cultures of primary chondrocytes to develop improved methods of transfection which combine high transfection efficiency with high levels of transgene expression. Existing methods of transfection of primary chondrocytes result in a transfection efficiency of 10-20%. We have

created vectors which show stable transfection efficiencies of 30-50% at relatively low selection (selection of transfected cells in 10μg/ml antibiotic). Advantageously, virtually 100% of transfected cells that are positive for antibiotic selection also express the gene of interest.

Plasmid vectors designed for stable expression of foreign proteins in eukaryotic cells typically contain two separate expression cassettes, one for the protein of interest and another for a selectable marker such as the Tn5 *neo* gene. Linearisation of the circular vector DNA takes place during genomic integration. If translation happens within the expression cassette for the protein of interest leaving the selectable marker cassette intact, the transfected cells will survive selection but will not express the foreign protein, an event more likely to occur with larger proteins as the open reading frame represents a greater proportion of the plasmid. Loss of protein expression with time can also occur as the selective pressure is only exerted on the drug resistance marker. These problems can be overcome if the two genes are contained within a single transcription cassette designed to produce a bicistronic mRNA, linking expression at the RNA level. Eukaryotic cells do not normally translate the second gene efficiently but can be directed to do so by certain sequences called Internal Ribosome Entry Site (IRES) which, as the name suggests, allow eukaryotic ribosomes to initiate translation further down the mRNA in a cap-independent manner.

We have used a new bicistronic vector, pIRES, to bring our test gene of interest, for example and not by way limitation, β-galactosidase and the antibiotic resistance gene under the same promoter (for example and not by way of limitation, cytomegalovirus (CMV), elongation factor 1 (EF-1) or ubiquitin promoters) to enhance gene expression in almost 100% of stable, antibiotic resistant cells. A relatively new antibiotic, blasticidin S, is used instead of more conventional antibiotics (eg neomycin), for quicker selection

of stably transfected cells. It is a potent translational inhibitor in both prokaryotic and eukaryotic cells. Resistance to blasticidin is conferred by the products of the BSD gene from Aspergillus terreus (Kimura, M., et al. (1994) *Biochim. Biophys.* ACTA 1219: 653-659).

It is therefore an object of the invention to develop new methods for the transfection of cells.

It is a further object of the invention to develop new methods for transfection of cells that additionally provides for enhanced transgene expression.

It is a further object of the invention to provide new vectors for use, particularly but not exclusively, in tissue engineering.

According to a first aspect of the invention, there is provided an isolated nucleic acid molecule comprising at least the functional part of the blasticidin resistance (BS) gene, or homologue thereof, linked via a recognition sequence, to at least one selected gene.

Preferably the isolated nucleic acid molecule is a vector adapted for the transfection of a cell and the expression of the gene encoded by the vector. Transfection may be for any desired purpose such as for tissue engineering, in vitro tissue component (connective tissue) characterisation, in vitro drug testing, transgenic tissue or "knock-out" tissue development for in vitro study of gene function or expression and the like. Vector is construed as hereinbefore described and reference herein to a vector includes any suitable presentation of a nucleic acid molecule.

Preferably the recognition sequence comprises at least the IRES sequence or a functional part, or homologue thereof. The recognition sequence acts as a stabilising agent to allow stabilisation of transfected cells.

The BSD gene acts as a highly efficient selection agent, to allow rapid selection of transfected cells, killing non-transfected chondrocytes for example within days. This has a number of advantages in particular rapid selection enables the period in which cells are grown in culture to be shortened, minimising the chance of dedifferentiation. It is active at low concentrations and it is inexpensive. We believe that it does not down regulate transcription of adjacent genes in the expression vector.

We have moreover enhanced the selective marker effect by combining in a bicistronic vector as a single cassette recognition sequence. This effectively eliminates false positives in which transfected cells express drug resistance but not the protein of interest, as can occur with conventional dual cassette vectors. Here we have combined the advantages of bicistronic constructs with rapid antibiotic selection and a strong viral/eukaryotic promoter to produce a plasmid capable of guaranteed high-level, long-term protein expression.

The selected gene may be the same as a gene endogenous to the cell to be transfected or to any other tissue or species, and intended for (over)expression thereof, or may be antisense for a gene endogenous to the cell and intended for inhibiting expression thereof by mRNA "knock-out" destroying mRNA or by gene "knock-out" for complete gene deletion.

The vector may comprise any gene which it is desired to express, for therapeutic, analytical, characterisation, testing or other purpose. Therapeutic genes include protective genes such as tissue inhibitor of metalloproteinases (TIMP) such as TIMP-1 and TIMP-3 for overexpression; antisense sequences

against potentially damaging genes such as matrix metalloproteinases, eg MMP-1, MMP-13, aggrecanases and the like for mRNA knock-out, inhibiting cartilage breakdown, and the like.

In the latter case, antisense sequences could be sequences from the open reading frame ORF of the gene and/or from the untranslated regions (UTR, usually 5' UTR). The use of UTR is generally more specific as these sequences are not conserved across different family members of a particular protein.

The vector preferably comprises suitable promoters, for example viral promoters such as cytomegalovirus (CMV) or 5'-LTR, or mammalian promoters such as elongation factor-1 (EF-1) and the like.

The vector may additionally comprise a transfection reagent, giving efficient transfection of cells, for example commercially available agents such as FuGENE-6 and the like.

For use in overexpression or enrichment, the vector may also include tags for example peptide tags such as V5 and/or His to detect transfected gene and distinguish from endogenous gene; reporter gene such as LacZ gene (encoding for beta-galactosidase), luciferase gene or the like, demonstrating the effectiveness of enrichment of selected cells with the bicistronic vector.

In a preferred embodiment of the invention said adaptation comprises the inclusion of appropriate expression control sequences which optimise the expression of vector encoded nucleic acid molecule(s), preferably in a single sequence for example the 5'LTR or other promoter controls reporter (eg luciferase) expression or inserted gene expression, BSD gene expression etc.

In a further aspect of the invention there is provided a nucleic acid molecule or vector comprising at least the functional part of the blasticidin resistance gene, or homologue thereof, linked to a recognition sequence as hereinbefore defined, comprising additional restriction site(s) for insertion of a selected gene or gene sequence for transfection.

Preferably a vector as hereinbefore defined is as illustrated in the vector map of Figure A or B and/or comprises at least the gene sequence or a part thereof as listed in the gene sequence of Figure C or D. The vectors of Figures A and B include additional restriction sites as hereinbefore defined for insertion of a specific gene (not shown). In particular the vector of Figure B includes a restriction site Sna B1, at the 5' end after the Xho 1 site of the existing MCS to facilitate insertion.

A viral vector as hereinbefore defined, for example under the control of the 5'LTR or other promoter, may provide excellent long term expression and high stability levels. In some cases however it may be advantageous for transfection of mammalian cells to employ a viral vector as hereinbefore defined which is self-inactivating, for example the retroviral 5'LTR promoter becomes inactivated and replaced by that of the inserted promoter/gene. This ensures that inserted genes are transcribed under the inserted promoter only, with inactivation of any other functional promoters which may be introduced for example viral promoters for additional gene sequences intended to facilitate or enhance transfection and ensures long term expression and high stability levels.

It will be apparent to one skilled in the art that said adaptation relates to a vector conventionally adapted for expression in either a prokaryotic cell and/or a eukaryotic cell. For example, and not by way of limitation, said adaptation comprises the provision of constitutive, inducible, or repressible promoter

elements; and/or the provision of polyadenylation control sequence for optimal expression in a eukaryotic cell; and/or the provision of termination sequences that facilitate the efficient expression of said vector in a prokaryotic cell; and/or the provision of selectable markers to allow the selection of said vector in a prokaryotic and/or eukaryotic cell.

In a further aspect of the invention there is provided a method for the preparation of a vector as hereinbefore defined comprising inserting the BSD gene in the absence of its promoter downstream of a recognition sequence in a suitable vector as a single cassette together with a selected gene also in the absence of its promoter.

The method employs isolation and insertion techniques as known in the art, in particular employing PCR with selection of suitable primers for deletion of gene sequences and insertion into vectors as desired.

In a further aspect of the invention there is provided a non-replicative virus comprising a vector as hereinbefore defined. Preferably a non-replicative virus is selected from adenovirus, retrovirus, parvovirus, herpesvirus, adeno-associated viruses, lentivirus and the like, preferably retrovirus such as moloney murine leukaemia virus (MoMuLV). The virus is packaged in conventional manner for example in packaging cell lines as known in the art.

A viral vector suitably comprises means to enhance fusion of virus with host plasma membrane in a cell to be transfected, in particular in fusing with cells which lack membrane receptor sites recognised by the virus. Suitable means may include the presence of proteins such as VSV-G coat protein and the like.

In a further aspect of the invention there is provided a method of transfecting a cell comprising;

i) incubating cells under conditions conducive to the introduction and maintenance of an isolated nucleic acid molecule as hereinbefore defined;

ii) introducing the isolated nucleic acid molecule into the cell;

iii) exposing the cells to blasticidin at a concentration sufficient such that at least those cells including the nucleic acid molecule are resistant to the blasticidin; and, optionally,

iv) culturing said cells containing said nucleic acid molecule; and, optionally, further still,

v) storing the cell culture prior to use.

Transfection of cells may be accomplished by stable or transient transfection as ehreinbefore defined, wherein the transfecting DNA integrates into the genome of the host cell or is maintained extrachromosomally or episomally.

The method of the invention is suitable for transfection of cells by any suitable medium, using any known technique, for example chemical transfection as the nucleic acid molecule via calcium phosphate precipitation, DEAE dextran mediated transfection, electroporation, direct injection through the cell membrane, or simply by suspension in culture; or viral transfection as a viral based vector comprising the nucleic acid molecule, such as adenovirus, retrovirus, parvovirus, herpesvirus, adeno-associated viruses, lentivirus vector and the like, preferably retrovirus vector.

Cells may be incubated under any conditions conducive to transfection, as known in the art. In a particular advantage of the invention we have found that

incubation of mitotically inactive cells which divide slowly in the presence of growth factors facilitates viral uptake, presumably but not exclusively by expanding the cells.

We have found that the method of the invention provides high transfection efficiencies of the order of 90% and above, and may be optimised at substantially 100%, whereby the transfection may be substantially time independent, ie expression remains constant and does not fall away. Optimisation may include the selection of a specific combination of transfection method, vector type and the like for a particular purpose, for example selection of retroviral transfection method, in particular for inhibition of gene expression by transfecting antisense material and the like into chondrocytes pre-incubated in the presence of growth factors or the like.

The cells may be exposed to blasticidin in any desired selectively effective amount and time-concentration profile, for example constant or increasing profile, and suitable amounts and profiles may be determined in known manner. Preferably exposure is in an amount of at least 10 μg/ml, and may be in excess of 50 μg/ml.

It will be apparent to one skilled in the art that genetically engineered cells/tissues according to the invention are useful in the provision of cells/tissues for replacement of cells/tissues affected by an inherited genetic disorder with a view to replacing/correcting the defective gene with a wild-type gene and thus reducing and/or reversing the detrimental effects of said genetic disorder.

Furthermore, as mentioned above, transfection of cells has facilitated the study of gene function and, in particular, how in multi-cellular organisms, tissue differentiation and organogenesis is regulated. Cell transfection has enabled

the study of the *in vivo* function of genes via the production of transgenic animals and plants. Transgenic animals and plants are organisms into which a gene or genes from another species, a transgene(s), has been incorporated. The gene thus introduced is not naturally found in the transgenic organism. Briefly the transfection technique involves the injection of nucleic acid into a fertilised egg (this technique has been particularly successful in mice). The injected eggs are then returned to a surrogate mother and the pregnancy allowed to go to term. Some of the pups that survive gestation carry the transgene. The expression of the transgene can then be followed during growth and development of the transgenic organism.

Transgenic techniques have also been used to target specific mutations. This became possible with the establishment of mouse embryonic stem cell cultures (embryonic stem cells are totipotent cells derived from an early mouse embryo which have the potential to develop into a complete embryo including extra-embryonic tissue). DNA is introduced into stem cells to create transgenic stem cells. These are then combined with embryos removed from a mouse to create chimeric embryos containing both the transgenic stem cells and cells originating from the embryo. The chimeric embryo is reintroduced into a surrogate mother and the embryos allowed to develop to term.

The specific inactivation of genes in a knock out requires the provision of a vector including the gene to be knocked out which has been genetically modified such that homologous recombination between the modified gene and the gene to be mutated results in the partial or entire deletion of the gene of interest. The effects of the loss of gene function, providing it is not lethal to the foetus, can be monitored during growth and development.

A recent strategy to interfere with the expression of a gene is that of antisense technology. In brief this strategy involves the use of a DNA or RNA molecule that is complementary to a region of a selected gene and is able to hybridise

(bind) under physiological conditions to the targeted nucleic acid to prevent either transcription of the gene or translation of the mRNA encoded by the gene. The antisense molecule is often a short oligodeoxynucleotide (ODN). However the molecule may be an oligodeoxyribonucleotide, or a modified oligodeoxynucleotide, or a modified oligodeoxyribonucleotide; each of which are able to hybridised to a selected part of a gene, or mRNA, under physiological conditions. The modifications to oligodeoxynucleotides will be apparent to one skilled in the art. The exact region of the nucleotide sequence of the gene to which the antisense molecule is designed can be empirically determined. However it is common practice to design oligodeoxynucleotides to the 5' region of the gene (to interfere with transcription initiation) or the mRNA (to interfere with translation). As stated before, the region of the gene to which the antisense molecule is directed is determined by the efficiency with which the antisense molecule suppresses the gene of interest. Contrary to the above this may be the 3' region and is determined experimentally. The length of the ODN also has to be determined experimentally. Typically ODNs are 20-30 nucleotides in length but may be much longer.

We have used the method described herein to generate cells/tissues inactivated or deleted for selected genes and analysed the effects of loss of said gene on cells/tissues in culture. This technique removes the need to generate transgenic animals for analysing the effects of gene deletion on cell/tissue function and thereby provides an *in vitro* model for analysis. Transgenic technology currently has many ethical problems attached to it. The technique hereindescribed obviates the need to use embryos or stem cells to generate transgenic animals, instead transgenic tissues are produced in culture. Moreover deletion of many genes in transgenic animals can be fatal developmentally whereby they are not useful for the above purposes. Transgenic tissue provides a simpler system in which to observe the effects of genes and model the effects of therapeutic agents and the like.

Preferably the method of transfecting a cell as hereinbefore defined is to inactivate or delete at least one selected gene, or at least the effective part thereof, in or from the genome of at least one cell/tissue.

In a further aspect of the invention there is provided a method of transfecting at least one cell to inactivate or delete at least one selected gene, or at least the effective part thereof, in or from the genome of at least one cell/tissue comprising;

i) introducing at least one isolated nucleic acid molecule comprising at least the functional part of a selectable marker gene, or homologue thereof, linked via a recognition sequence, to at least one selected gene or antisense material into at least one cell, wherein the nucleic acid molecule is modified by means of the selected gene or antisense material to facilitate the inactivation of or deletion, or at least partial deletion, of at least one selected gene;

ii) incubating said cell under conditions conducive to the inactivation of or deletion of said gene;

iii) cloning said cell; and

iv) analysing said cloned culture to establish the loss of the mRNA produced by the gene or the loss of the gene.

Preferably the selectable marker gene is blasticidin resistance gene or a functional part thereof as hereinbefore defined. Preferably the nucleic acid molecule is a vector according to the invention as hereinbefore defined.

Preferably inactivation or (partial) deletion of a specific gene is either by homologous recombination to delete the specific gene or by the introduction of antisense sequences upstream of the recognition and selection sequences, preferably the IRES/BSD sequence in the IRES vectors, in order to downregulate the specific mRNA for that gene.

In a preferred method of the invention said cell is a differentiated cell.

It will be apparent to one skilled in the art that methods exist to analyse the inactivation of or deletion of a gene(s) from the genome of an organism. These include by example and not by way of limitation, Southern blotting to identify **R**estriction **F**ragment **L**ength **P**olymorphism (RFLP) in the or around the gene(s) of interest; **P**olymerase **C**hain **R**eaction (PCR) assays to identify deletions; *in situ* PCR to identify chromosome deletions; **F**luoresence **I**n **S**itu **H**ybridisation (FISH) to identify chromosome deletions; expression analysis using northern blots to show the lack of a selected mRNA; western blots to show the lack of a polypeptide(s) encoded by a gene(s) deleted.

In a preferred method of the invention said cell/tissue carrying said inactivated or deleted gene(s) is further analysed to determine the effects of said inactivation or deletion on cell/tissue function.

In a further aspect of the invention there is provided a method for incubating mitotically inactive cells for transfection with any vector as known in the art comprising culturing in the presence of growth factor for a suitable period prior to introduction of the vector.

In a further aspect of the invention, there is provided at least one cell or tissue transfected by the nucleic acid molecule or vector or method according to the invention as hereinbefore defined.

In a further aspect of the invention there is provided a cell line or tissue cloned or cultured from at least one cell transfected according to the invention as hereinbefore defined.

In a further aspect of the invention there is provided a method for the selection of a cell, cell line or tissue transfected according to the invention with

substantially 100% efficiency, or detecting the efficiency of transfection comprising exposing the cell, cell line or tissue to blasticidin and selecting cells which remain viable, or determining cell death. The method may be used at any time after the transfection has taken place and may be used for periodic post transfection selection or for verification of results obtained with transfected cells, for example in antisense studies for mRNA or gene knock out.

In a further aspect of the invention there is provided a therapeutic composition comprising cells/tissues transformed with at least one nucleic acid molecule according to any previous aspect or embodiment of the invention. The therapeutic composition is preferably for use in tissue engineering; or for use in cosmetic surgery; or the composition comprises tissue transformed by gene "knock-out" as hereinbefore defined and is for use in identifying the role of genes in healthy and diseased tissue.

In a further aspect of the invention there is provided the use of a transfected cell or cell line or tissue or composition or a cell line, tissue or composition cloned or cultured therefrom, in tissue engineering, in particular in therapeutic or cosmetic tissue engineering, or in knock out therapy or in vitro study, in particular in antisense technology.

Therapeutic tissue engineering includes for example the replacement of diseased or damaged tissue. Conditions which would benefit from therapeutic tissue engineering include by example, and not by way of limitation, arthritis and the replacement of joints; skin grafting for burns victims or injuries resulting in severe contusions; replacement coronary arteries.

Conditions which would benefit from cosmetic tissue engineering include by example and not by way of limitation, rhinoplasty.

Identification of the role of genes is a particularly important area which is potentially of major significance in developing and applying transgenic tissue. The composition of the invention may be useful in providing a rapid and simplified route for identifying target genes for potential therapy or for use to provide modelled diseased tissue by increasing or decreasing expression of genes known to play a role in disease processes (such as specific proteases in arthritis).

In a further aspect of the invention, there is provided a method of treatment comprising;

i) providing cells/tissues transfected with at least one nucleic acid molecule according to the invention as hereinbefore defined;

ii) surgical administration of said cells/tissues to a patient to be treated; and optionally;

iii) monitoring the status of said cells/tissue by the patient.

The invention will now be illustrated in non-limiting manner, by example only and with reference to the following figures

Figure 1 is a graphical representation of cell death of untransfected cells after exposure to blasticidin or neomycin;

Figure 2 shows reporter gene LacZ expression by articular chondrocytes after transient transfection;

Figure 3 shows reporter gene LacZ expression by articular chondrocytes after stable transfection in the presence of blasticidin; and

Figure 4 shows reporter gene LacZ expression by adult chondrocytes after stable transfection in the presence of blasticidin.

Materials and Methods

Isolation of chondrocytes from nasal/articular cartilage

Full-thickness, bovine articular or nasal cartilage was harvested aseptically from adult animals (16-20 months). Cartilage was minced finely in PBS and the chondrocytes isolated from cartilage by sequential digestion at 37°C for 15 min with 1 mg/ml testicular hyaluronidase (Sigma), 0.25% (w/v) trypsin (Gibco BRL) for 30 min, and 1.5 mg/ml clostridial collagenase (Sigma) overnight on an orbital shaker. Isolated chondrocytes were centrifuged and the resulting pellet was resuspended in complete DMEM containing 10% FCS, bFGF (10 ng/ml; PeproTech) and the antibiotics penicillin and streptomycin (Gibco BRL). The cells were seeded in 6-well tissue culture plates (Greiner) at a density of $1.5x10^6$ cells/well.

Chondrocyte seeding on PGA scaffold and construct culture

Cultured chondrocytes were trypsinised, centrifuged and resuspended in complete DMEM containing 10 μg/ml insulin (Sigma), 50 μg/ml ascorbic acid (Sigma) and penicillin and streptomycin. PGA scaffolds produced as previously described [Freed et al., 1994] were pre-soaked in medium for 24 h at 37°C. The scaffolds were then seeded with chondrocytes in well-mixed spinner flasks as previously described [Vunjak-Novakovic et al., 1998]. Briefly, 2 scaffolds per flask were threaded onto a wire gauge and seeded with 60 ml of cell suspension containing $5x10^5$ cells/ml at 50 rpm. After 3 days, cell-polymer constructs were transferred to 35mm dishes coated with a thin film of 1% agarose. The dishes were placed on an orbital shaker set at 75 rpm and cultured for up to 8 weeks at 37°C/5% CO_2. Medium was replaced every 2-3 days.

Biochemical analyses of tissue-engineered cartilage

Samples of natural or engineered cartilage were weighed then digested with proteinase K in the presence of protease inhibitors [Hollander et al., 1994]. Collagen type II was quantified by using proteinase K-digested samples in a monoclonal antibody-based inhibition ELISA [Hollander et al., 1994]. Total collagen content was determined from the hydroxyproline content after acid hydrolysis (6 N HCl at 115°C for 18 h) and reaction with p-dimethylaminobenzaldehyde (BDH) and chloramine T (BDH) by using a ratio of hydroxyproline to collagen of 0.1 (Hollander et al, 1994). Sulfated glycosaminoglycan (GAG; the polysaccharide component of proteoglycan) content was determined spectrophotometrically after reaction with dimethylmethylene blue dye (BDH) by using bovine chondroitin sulfate as a standard [Creemers et al., 1997].

Construction of plasmid expression vectors

The bicistronic pIRES/neo plasmid expression vector (Clontech) was used as a backbone for inserting various genes of interest. Initially, the resistance gene for neomycin was replaced with the resistance gene for blasticidin. The blasticidin resistance gene was amplified by PCR from pcDNA6/V5-His plasmid (Invitrogen) using the primers 5'GCCGCCCCCGGGATAATTCCTGCAGCCAATATGGCCAAGCCTTTGTCTCAAG3', incorporating SmaI/XmaI site (underlined) and

5'-GCCGCCTCTAGATTAGCCCTCCCACACATAACCA-3', containing XbaI site (underlined). The PCR product was digested with SmaI and XbaI and cloned between the XbaI and SmaI sites of pIRES/neo to generate pIRES/BS. A control vector based on pIRES/BS was constructed for the evaluation of the transfection efficiency. The marker gene used was β–

galactosidase gene (LacZ). It was amplified using PCR from pcDNA6/V5-His/LacZ (Invitrogen) with the primers
5'-GCCGCCGCCGCGGCCGCCACCATGATAGATCCCGTCGTTTTACAAC-3',
incorporating NotI site (underlined) and
5'-GCCGCCGGATTCTTATTTTTGACACCAGACCAACTG-3', incorporating BamHI site (underlined). The PCR product was digested with NotI and BamHI and cloned between the NotI and BamHI sites in the multiple cloning sites of pIRES/BS. Human tissue inhibitor of metalloproteinases-1 (TIMP-1) was amplified using PCR from a shuttle vector (courtesy of Dr. Norman McKie, University of Newcastle upon Tyne) with the primers;
5'-GCCGCCAAGCTT GGATCCGCCGCCACCATGGCCCCCTTTGAGCCCC-3',
incorporating HindIII and BamHI sites, respectively (underlined) and
5'-GCCGCCCTCGAGGAATTCGGCTATCTGGGACCGCAGG-3',
incorporating XhoI and EcoRI sites, respectively (underlined). The PCR product was digested with HindIII and XhoI and subcloned in pcDNA6/V5-His. The correct sequence of the amplified TIMP-1 was confirmed by sequencing. TIMP-1 gene fused to V5-His tags was then cloned from pcDNA6 by digestion with BamHI and PmeI and was subcloned in the multiple cloning site of pIRES/BS between BamHI and a polished (blunted) BstXI site. The polishing was carried out using Pwo polymerase to digest the 5'-overhangs of BstXI cohesive end so that it becomes compatible with the 3'-blunt end of TIMP-1 generated by PmeI. An anti-sense for the 5'-UTR sequence of the bovine TIMP-3 gene was synthesised by Dr. Arthur Moir (Krebs Institute, University of Sheffield). The 49 nucleotide sequence was flanked by a restricted 5'-NotI site and 3'-BamHI site for insertion into the multiple cloning site between NotI and BamHI sites in the pIRES/BS.

Chemical transfection of chondrocytes and selection of stable cells

Transfection is illustrated in Figures 1 to 4 in which

Figure 1 shows cell death for treatment of chondrocytes with antibiotics. Untransfected adult bovine articular chondrocytes were cultured in monolayer with 500µg/ml neomycin, 10µg/ml blasticidin or no antibiotic (Control). At each time point cells from all culture conditions (3 wells in each case) were enzymatically isolated and the viable cells were counted following trypan blue exclusion. By day 12 cells in control wells had undergone some proliferation whereas no viable cells could be detected in blasticidin-treated cultures. In neomycin-treated wells the cell number had fallen only slightly by day 12 compared to day 0.

Figure 2 shows transient transfection of adult bovine articular chondrocytes. Chondrocytes were cultured in monolayer for 3 days with (LacZ) or without (control) chemical transfection of the LacZ marker gene using a newly established methodology. The cultures were stained for ß-galactosidase using the standard technique. Cells staining positive always represented 5-10% of the total cell population.

Figure 3 shows stable transfection of adult bovine articular chondrocytes. Chondrocytes were cultured in monolayer for 3 days with chemical transfection of the LacZ marker gene in the pcDNA6 vector, using a newly established method. Stable transfection was then achieved by selecting positive cells with the antibiotic "blasticidin" at different concentrations for 12 days. After selection, all cultures were stained for ß-galactosidase using the standard technique. Blasticidin selection resulted in a marked increase in the proportion of positive cells compared to the "No Blasticidin" control. However this proportion never reached more than about 50% of the total cell population.

Figure 4 shows complete expression of LacZ by stable transfected adult bovine chondrocytes. Chondrocytes were cultured in monolayer for 3-5 days with chemical transfection of the LacZ marker gene in the pcDNA6 vector, as in Materials and Methods. Selection for positive cells with balsticidin at 40µg/ml achieved almost 100% stable transfection efficiency in 7 days.

Chondrocytes freshly added to 6-well tissue culture plate were transfected with plasmid DNA combined with FuGENE 6 reagent (Boehringer Mannheim). Briefly, 6µl of FuGENE 6 was mixed with 2µg of DNA in 100µl of serum free medium for each well. The FuGENE 6 reagent:DNA complex was added to the cells while in suspension ($1.5x10^6$ cells/well; 2 ml complete medium/well containing bFGF, see above). Cells were analysed for gene expression at least 3-days after transfection. Initially, the LacZ reporter gene vector was used to evaluate the efficiency of transfection. The experiments revealed an efficiency of 5-20%. The selection for stable transfected cells started 3-5 days after transfection using 40µg/ml blasticidin (Invitrogen) for 1 week. The cells were switched to a lower dose of blasticidin at 20µg/ml for 4 days then to a lower dose of 10µg/ml for another 4 days. The first week of selection yields approximately 90% LacZ positive cells which need to be stabilised gradually using the lower concentrations of blasticidin.

RESULTS

Empirical experiments were carried out to determine the effective dose of blasticidin, or neomycin for comparison, required to kill all the untransfected chondrocytes over the shortest period of time in comparison to neomycin. The results demonstrate that blasticidin achieved complete cell death within 12 days (Figure 1).

The expression vector, pcDNA6/LacZ (Invitrogen) which encodes the LacZ gene and bsd gene each under separate promoter was used initially to test the ability of the chemical transfection reagent, Fugene6, to transfect chondrocytes. The transfection method repeatedly achieved a transient transfection rate of 5-10% (Figure 2). After 12 days of selection with blasticidin, 40-50% of the resistant cells were LacZ positive (Figure 3). This

is not unexpected as the LacZ and antibiotic resistance genes are expressed under separate promoter and so antibiotic selection will stabilise the resistance gene rather than the gene of interest.

A pIRES vector encoding the LacZ and bsd genes under one promoter was constructed (see Materials & Methods) and named, pIRES/BS/LacZ. Adult bovine chondrocytes were initially transfected with the vector and cultured in monolayer with 40 μg/ml blasticidin for 7 days. After that time about 99% of the cells were positive for LacZ (Figure 4), demonstrating effective enrichment of the positive cells with a bicistronic vector.

Transfected cells (enriched or non-enriched) were seeded onto the PGA scaffold and kept in culture for 40 days.

Retroviral transduction of chondrocytes and selection of stable cells

Retroviral vectors based on IRES sequence and blasticidin are also used in the method of the invention. These vectors are combined with the use of proliferative growth factors like b-FGF, TGF-β, IGF-I, BMP-2, BMP-4, and/or BMP-7. The method of the invention is applicable to other cell types utilising the developed transfection technology.

Retroviruses are currently the main method of choice for the introduction of genes in gene therapy trials as they facilitate efficient and stable incorporation of genes into the host genome. However retroviral transduction requires that the target cells are mitotically active. This has precluded their use for the efficient introduction of genes into chondrocytes as these cells divide very slowly. However we have shown this problem is overcome by the treatment of the cells with basic Fibroblast Growth Factor (bFGF) as outlined above (this has the advantage of expanding the isolated chondrocytes while at the

same time facilitating retroviral uptake), together with the use of a modified replication deficient retrovirus.

Construction of retroviral transfection vectors

Efficiency of transduction of chondrocytes was determined using a commercially available (Clontech UK) retroviral transduction system, the "Pantropic Retroviral Expression System" incorporating the luciferase gene as a reporter. This system produces replication defective Moloney murine leukaemia virus (MoMuLV) derived retrovirus bearing the envelope glycoprotein of vesicular stomatis virus (VSV-G). This permits transduction of bovine cells and may increase transduction efficiency of human cells.

Initial experiments utilised retroviruses produced by co-transfection of GP-293 cells with the pLXRN vector containing the luciferase reporter under the control of the 5'LTR of the retrovirus and the pVSV-G vector which bears the gene for the VSV-G coat protein.

Retroviral transfection of chondrocytes and selection of stable cells

Bovine articular or nasal chondrocytes were isolated and cultured as outlined above in the presence of bFGF for 2-5 days. The cells were then infected with the various titres of the reporter virus and expression of luciferase monitored for up to 8 weeks.

Bovine chondrocytes were very efficiently transduced with the reporter virus, expression of luciferase increasing with increasing viral titre, although at very high viral titres the target cells were lysed. In the absence of antibiotic selection expression levels declined over the 6 weeks culture period but luciferase was still detectable at 6 weeks. When the transduced cells were

treated with G418 (the pLXRN vector contains the neomycin resistance gene), expression levels were stabilised. This stable expression level appeared to be independent of initial viral titre, suggesting the cells can only accommodate a restricted copy number of the target gene.

RESULTS

The pLXRN vector was modified by replacing the neomycin resistance gene and its promoter with the IRES sequence and the blasticidin resistance gene as described above (see Figs A and B for vector map and gene sequence), as single cassette with selected therapeutic genes or antisense material as described above, introduced at the multiple cloning site of the IRES/BS.

DISCUSSION

The use of bicistronic vectors with the selectable marker downstream of an IRES sequence effectively eliminates false positives in which transfected cells express drug resistance but not the protein of interest, as can occur with conventional dual cassette vectors. Here we have combined the advantages of bicistronic constructs with a strong viral/eukaryotic promoter to produce a plasmid capable of guaranteed high-level, long-term protein expression.

Blasticidin as a selectable marker has several advantages over such drugs as G418: it acts quickly to kill non-transfected chondrocytes within 7-12 days reducing the problem of dedifferentiation, it is active at low concentrations (5-10μg/ml) and it is inexpensive. There have been reports that neo gene can down regulate transcription of adjacent genes in the expression vector, an effect that would encourage using other selectable markers.

References

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CLAIMS

1. An isolated nucleic acid molecule comprising at least the functional part of the blasticidin resistance (BS) gene, or homologue thereof, linked via a recognition sequence, to at least one selected gene.

2. An isolated nucleic acid molecule as claimed in Claim 1 which is a vector adapted for the transfection of a cell and the expression of the gene encoded by the vector.

3. An isolated nucleic acid molecule as claimed in Claim 1 or 2 wherein the recognition sequence comprises at least the IRES sequence or a functional part, or homologue thereof.

4. An isolated nucleic acid molecule as claimed in any of Claims 1 to 3 which is a bicistronic vector wherein the BS and selected gene are comprised in a single cassette recognition sequence.

5. An isolated nucleic acid molecule as claimed in any of Claims 1 to 4 wherein a selected gene is the same as a gene endogenous to the cell to be transfected or to any other tissue or species or is be antisense for a gene endogenous to the cell.

6. An isolated nucleic acid molecule as claimed in any of Claims 1 to 5 wherein a selected gene is selected from a protective gene such as tissue inhibitor of metalloproteinases (TIMP) TIMP-1 and TIMP-3 for overexpression, and antisense sequences against potentially damaging genes such as matrix metalloproteinases, eg MMP-1, MMP-13, aggrecanases and the like for mRNA knock-out, inhibiting cartilage breakdown

7. An isolated nucleic acid molecule as claimed in any of Claims 1 to 6 wherein antisense sequences are sequences from the open reading frame ORF of the gene and/or from the untranslated regions (UTR) preferably 5' UTR

8. An isolated nucleic acid molecule as claimed in any of Claims 1 to 7 which comprises viral promoters such as cytomegalovirus (CMV) or 5'-LTR, or mammalian promoters such as elongation factor-1 (EF-1).

9. An isolated nucleic acid molecule as claimed in any of Claims 1 to 8 which additionally comprises a transfection reagent, giving efficient transfection of cells, preferably FuGENE-6.

10. An isolated nucleic acid molecule as claimed in any of Claims 1 to 9 which also includes tags for example peptide tags such as V5 and/or His to detect and distinguish transfected gene.

11. An isolated nucleic acid molecule as claimed in any of Claims 1 to 10 and illustrated in the vector map of Figure A or B and/or comprising at least the gene sequence or a part thereof as listed in the gene sequence of Figure C or D.

12. An isolated nucleic acid molecule as claimed in any of Claims 1 to 11 which is a self-inactivating viral vector.

13. Method for the preparation of an isolated nucleic acid molecule or vector as claimed in any of Claims 1 to 12 comprising inserting the BSD gene in the absence of its promoter downstream of a recognition sequence in a suitable vector as a single cassette together with a selected gene also in the absence of its promoter.

14. A non-replicative virus comprising a vector as claimed in any of Claims 1 to 13.

15. A method of transfecting a cell comprising;

i) incubating cells under conditions conducive to the introduction and maintenance of an isolated nucleic acid molecule as hereinbefore defined;

ii) introducing the isolated nucleic acid molecule into the cell;

iii) exposing the cells to blasticidin at a concentration sufficient such that at least those cells including the nucleic acid molecule are resistant to the blasticidin; and, optionally,

iv) culturing said cells containing said nucleic acid molecule; and, optionally, further still,

v) storing the cell culture prior to use.

16. Method as claimed in Claim 15 for transfection of mitotically inactive cells which divide slowly wherein incubation is in the presence of growth factors .

17. Method as claimed in Claim 15 or 16 characterised by high transfection efficiencies of the order of 90% and above, and preferably substantially 100%.

18. A method of transfecting at least one cell to inactivate or delete at least one selected gene, or at least the effective part thereof, in or from the genome of at least one cell/tissue comprising;

i) introducing at least one isolated nucleic acid molecule comprising at least the functional part of a selection gene, or homologue thereof, linked via a recognition sequence, to at least one selected gene or antisense material into at least one cell, wherein the nucleic acid molecule is modified by means of the selected gene or antisense material to facilitate the inactivation of or deletion, or at least partial deletion, of at least one selected gene;

ii) incubating said cell under conditions conducive to the inactivation of or deletion of said gene;

iii) cloning said cell; and

analysing said cloned culture to establish the loss of the mRNA produced by the gene or the loss of the gene.

19. A method for incubating mitotically inactive cells for transfection with any vector as known in the art comprising culturing in the presence of growth factor for a suitable period prior to introduction of the vector.

20. At least one cell transfected by the nucleic acid molecule or method as claimed in any of Claims 1 to 19.

21. A therapeutic composition comprising cells/tissues transformed with at least one nucleic acid molecule or with the method as claimed in any of Claims 1 to 19.

22. Therapeutic composition as claimed in Claim 21 for use in tissue engineering; or for use in cosmetic surgery; or wherein the composition

comprises tissue transformed by gene "knock-out" as hereinbefore defined and is for use in identifying the role of genes in healthy and diseased tissue.

23. A method of treatment comprising;

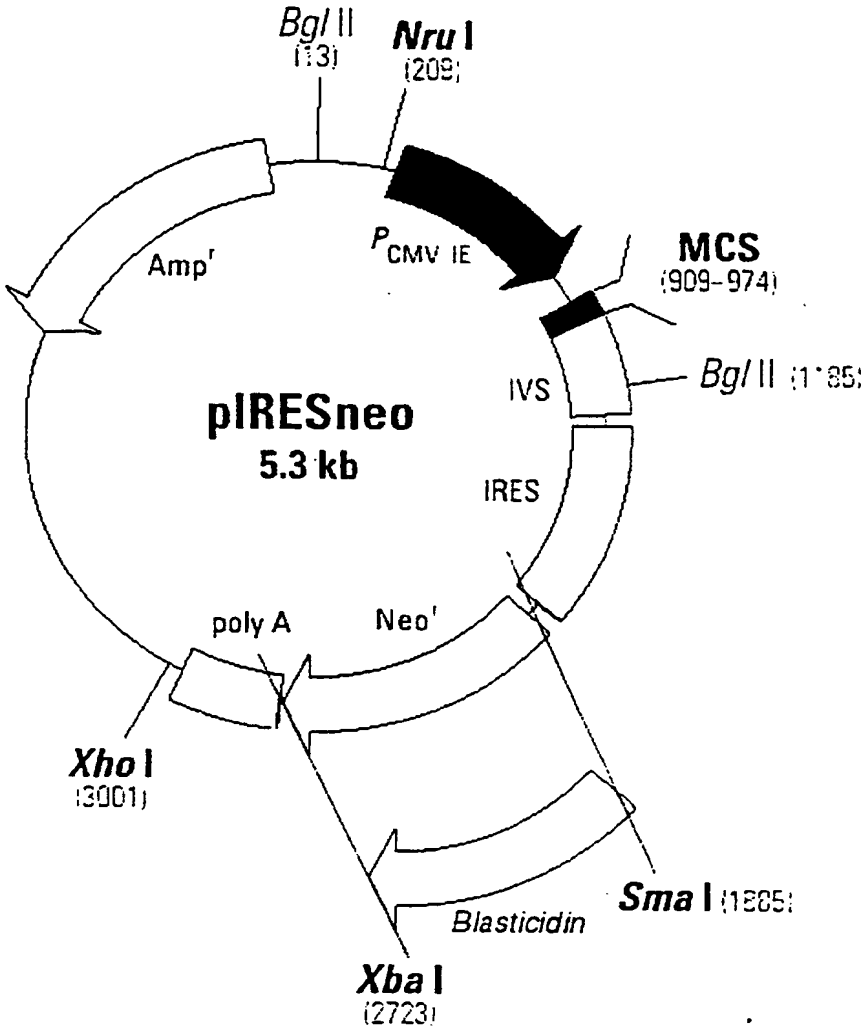
i) providing cells/tissues transfected with at least one nucleic acid molecule according to the invention as hereinbefore defined;

ii) surgical administration of said cells/tissues to a patient to be treated; and optionally;

iii) monitoring the status of said cells/tissue by the patient.

24. A nucleic acid molecule, vector, method transfected cell or tissue or composition or use thereof suibstantially as hereinbefore described or illustrated in the description, figures and/or examples

Figure A. Replacement of neomycin resistace gene of pIRES.neo with that of blasticidin to create vector pIRES/BS.



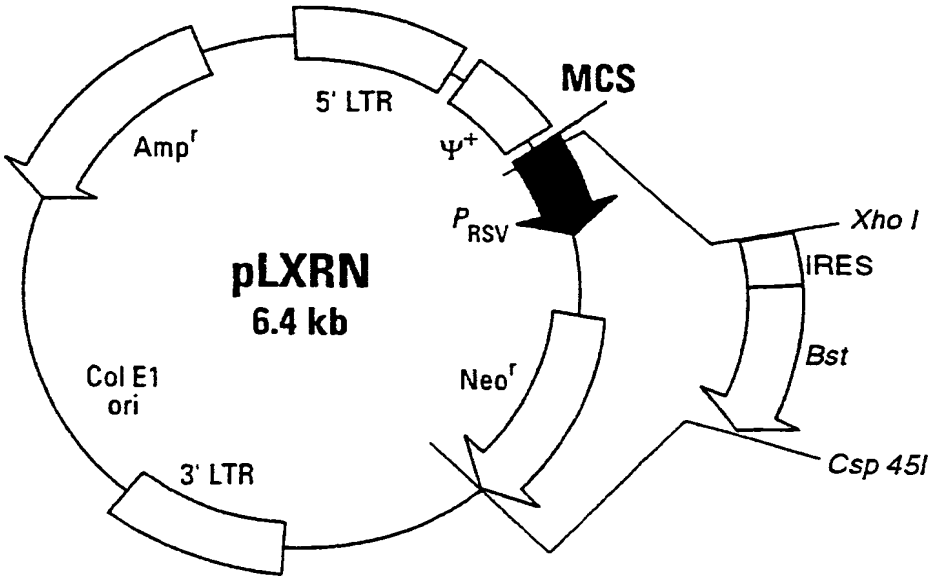


Figure B Replacement of RSV promoter and neomycin resistance gene of pLXRN with IRES and blasticidin resistance gene to create vector pLXIRES/BS

Figure C. Complete sequence of vector pIRES/BS

GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTAT
CTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGA
CAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGCGTTGACATT
GATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAA
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GGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC

Figure D Complete pLXIRES/BS sequence 6.3kb

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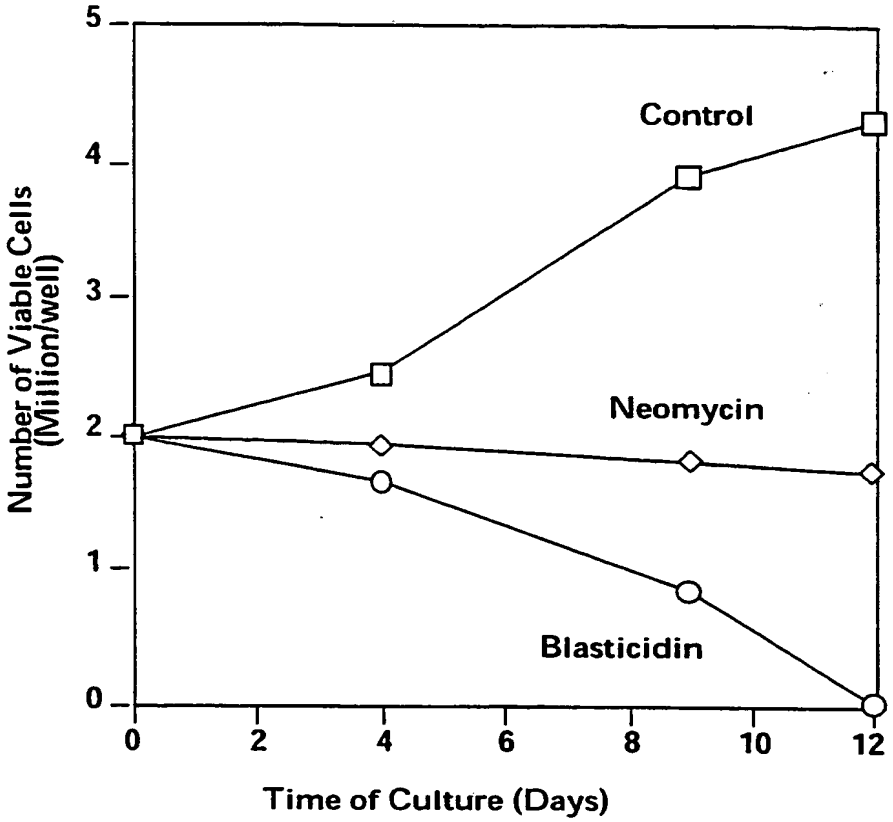

FIGURE 1: Cell death curve for treatment of chondrocytes with antibiotics

FIGURE 2: Transient transfection of adult bovine articular chondrocytes

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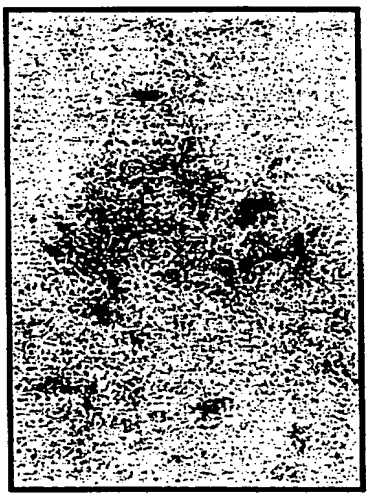
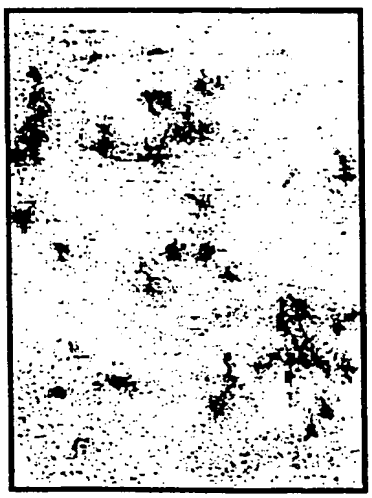
10 µg/ml Blasticidin



5 µg/ml Blasticidin



No Blasticidin

FIGURE 3:Stable transfection of adult bovine articular chondrocytes

Figure 4: Complete expression of LacZ by stable transfected adult bovine chondrocytes

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(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF SHEFFIELD** [GB/GB]; Western Bank, Sheffield S10 2TN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **HOLLANDER, Anthony, Peter** [GB/GB]; University of Bristol, Academic Rheumatology Unit, Avon Orthopaedic Centre, Southmead Hospital, Bristol BS10 5NB (GB). **BARKER, Michael, David** [GB/GB]; University of Sheffield, Division of Oncology [entity:amp] Cellular Pathology, Pathology Section, Medical School, Sheffield S10 2RX (GB). **KAFIENAH, Waeh** [PK/PK]; University of Sheffield, Division of Biochemical [entity:amp] Muscoloskeletal Medicine, Section of Human Metabolism [entity:amp] Clinical Biochemistr, y, Medical School, Beech Hill Road, Sheffield S10 2RX (GB).

(74) Agent: **MARKGRAAF PATENTS LIMITED**; The Crescent, 54 Blossom Street, York YO24 1AP (GB).

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(54) Title: CELL TRANSFECTION

(57) Abstract: An isolated nucleic acid molecule comprising at least the functional part of the blasticidin resistance (BS) gene, or homologue thereof, linked via a recognition sequence, to at least one selected gene; a method for the preparation thereof, a method for transfecting cells, a transfected cell and tissue comprising a transfected cell, a method for "knockout", a method for incubating mitotically inactive cells, a therapeutic composition comprising transfected cells or tissue, and the use thereof.

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 00/03462

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/78 C12N15/55 C12N15/63 C12N15/11 C07K14/81
C12N5/10 C12N15/86 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, STRAND, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
6 June 2001	22/06/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

2

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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